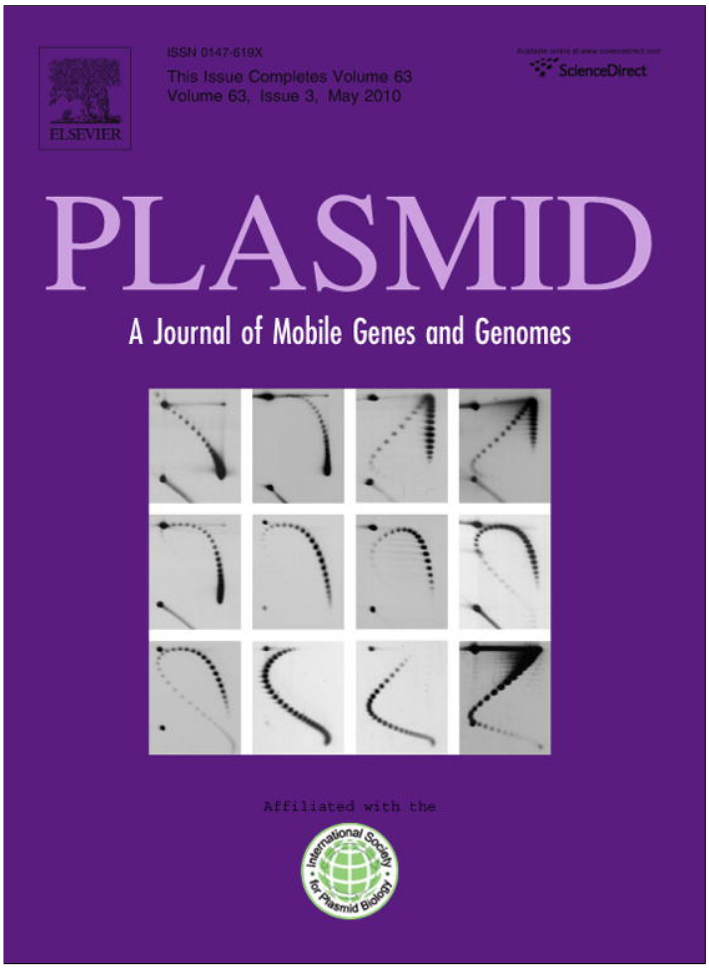

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Short Communication

Characterization of small ColE1-like plasmids conferring kanamycin resistance in *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Newport

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ABSTRACT

Multi-antibiotic resistant (MR) *Salmonella enterica* serovars Typhimurium and Newport are an increasing concern in human and animal health. Many strains are known to carry antibiotic resistance determinants on multiple plasmids, yet detailed information has been scarce. Three plasmids conferring kanamycin (Kan) resistance were isolated and nucleotide sequences were determined. Two Kan^R plasmids from *Salmonella* Newport strains, pSN11/00Kan and pSN02/01Kan, were found to be identical and were 5698 bp in size. Plasmid pG7601Kan from *Salmonella* Typhimurium phage type U302 strain G7601 was 3208 bp, and was the same as the previously reported pU302S from another U302 strain G8430. All three plasmids carried identical *aph*(3′)-I genes. The plasmids were ColE1-like, containing RNA I/RNA II and the *rom* gene. Plasmids pSN11/00Kan and pSN02/01Kan also carried mobilization genes *mobC* and *mobABD*, similar to those of the pColK-K235 and pColD-157 plasmids from the colicinogenic *Escherichia coli* strains. All three plasmids were stable without kanamycin selection for ~100 generations.

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Multi-antibiotic resistant (MR) isolates of *Salmonella enterica* strains can carry resistance genes on plasmids or on the chromosome, where they are present singularly or arranged as cassettes in "integrons". Many *Salmonella* strains have been shown to carry multiple plasmids of varying sizes, including the virulence plasmid of approx. 50–100 kb (Rychlik et al., 2006). Most research has focused on characterization of the large plasmids in these strains (60–100 kb or larger) (Carattoli et al., 2005, 2006). Relatively few reports focused on the low molecular weight plasmids, which were estimated to be present in about 10% of the *Salmonella* field strains (data not shown). These small plasmids can carry genes encoding a wide variety of biological functions, such as restriction–modification systems, retron reverse transcriptase, O-antigen, and antibiotic resistance (Rychlik et al., 2006). However, most of the reports on the small plasmids were focused on the potential of plasmid profiling as molecular typing tools for *Salmonella* species (Gregorova et al., 2002, 2004; Millemann et al., 1995; Threlfall et al., 1994).

Previously we characterized two plasmids from a MR *Salmonella* Typhimurium phage type U302 strain G8430 (Chen et al., 2007). The large plasmid pU302L was 84.5 kb in-length, and encoded 11 genes involved in resistance to seven antibiotics and the *mer* operon which confers resistance to mercury ions. The small 3.2 kb plasmid pU302S was ColE-1 like, and carried the *aph*(3′)-I gene conferring kanamycin resistance (Kan^R) (Chen et al., 2007). A quick survey of the available *Salmonella* strain collections indicated that many kanamycin-resistant isolates also carry ColE-1 like plasmids (unpublished results; C.-Y. Chen

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and R. Lindsey). Three kanamycin-resistant ColE+ strains were further investigated: one was serovar Typhimurium and two were *Salmonella enterica* subsp. *enterica* serovar Newport. The *S.* Typhimurium strain G7601 (phage type U302) was a human clinical isolate obtained from the Center for Disease Control and Prevention (Atlanta, GA) and was resistant to 14 antibiotics tested (Ampicillin, Carbenicillin, Cefuroxime, Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Nalidixic acid, Neomycin, Streptomycin, Sulfamethoxazole, Tetracycline, Tobramycin, and Trimethoprim) (Briggs and Fratamico, 1999, and unpublished data). The two *Salmonella* Newport strains SN11/00 and SN02/01 were veterinary diagnostic isolates from the National Antimicrobial Resistance Monitoring System (NARMS) and were resistant to 10 antibiotics (Amoxicillin/Clavulanic acid, Ampicillin, Cefoxitin, Ceftiofur, Cephalothin, Chloramphenicol, Kanamycin, Streptomycin, Sulfamethoxzole, and Tetracycline) and 11 antibiotics (all of the above plus Trimethoprim/sulfamethoxazole), respectively.

All bacterial cultures were maintained on Luria–Bertani (LB) agar or broth (Becton, Dickinson and Company, Sparks, MD) at 37 °C. Total plasmid DNA was purified from the original *Salmonella* strains, then transformed into *Escherichia coli* DH5α and selected for Kan^R transformants on LB agar supplemented with kanamycin A 50 μg/ml (Sigma–Aldrich, St. Louis, MO). Plasmids were then purified from the *E. coli* host using Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA). Libraries were constructed by digesting the plasmids with either *Sau3A*I or *Nla*III, and cloned into pUC19 digested with *BamH*I or *Sph*I (New England Biolabs, Beverly, MA) using standard methods (Sambrook and Russell, 2001). DNA sequencing was performed using the BigDye Terminator kit (v. 3.1; Applied Biosystems, Inc., Foster City, CA) and DNA Analyzer 3730 or 3130 (Applied Biosystems, Inc.). Contigs were assembled using Sequencher DNA sequence assembly software (v. 4.8; Gene Codes Corp., Ann Arbor, MI). Gap closures were completed using primer walking with custom primers (Integrated DNA Technologies, Inc., Coralville, IA). Restriction digest patterns of the plasmids were verified against *in silico* patterns to ensure correct assembly. Assembled sequences were annotated using Artemis DNA sequence viewer and annotation tool (v. 10; Rutherford et al., 2000; http://www.sanger.ac.uk/Software/Artemis/). Protein and nucleotide sequences were searched against NCBI GenBank (database posted September 23, 2009) using BLAST programs (BLASTP 2.2.21+ or BLASTN 2.2.21+; Altschul et al., 1997; Zhang et al., 2000).

The Kan^R ColE1-like plasmid pG7601Kan from *S.* Typhimurium strain G7601 was 3208 bp in-length, and was identical to the previously sequenced plasmid pU302S from strain G8430 (GenBank Accession No. AY333433), thus the sequence was not deposited in the GenBank and will not be discussed further. The nucleotide sequences of the two Newport plasmids, pSN11/00Kan and pSN02/01Kan, were found to be identical: both were 5698 bp in-length, with a G + C content of 53%. These two Newport strains were isolated in different years from different regions of the United States as defined by the NARMS program (http://www.ars.usda.gov/Main/docs.htm?docid=6750) and did not appear to be clonal (data not shown). Only the sequence of plasmid pSN11/00Kan was deposited in GenBank (Accession No. GQ470395). The open reading frames (ORF) and their putative functions on the pSN11/00Kan are listed in Table 1. The relaxase MobA and the organization of the mobilization operon *mobCABD* belong to the MOB_{HEN} family of the ColE1 superfamily of mobilizable plasmids (Francia et al., 2004). The putative RNA and DNA features involved in replication initiation/termination, such as RNA I/II, *ori*V, *ori*T, and *cer*, were marked based on other annotated plasmids pU302S, ColE1, and pSFD10 (Table 2). There was a partial ORF (179 aa; nt 5114–5656 of pSN11/00Kan) located between *aph*(3′)-I gene and the RNA I region, which showed 100% identity to TnpA transposase (full-length 990 aa) of the Tn3 family of transposons (ref|YP_743743 and others), suggesting that the *aph*(3′)-I gene of the pSN11/00Kan may have come from the family of Tn3 transposons.

Regions of similarity between plasmid pSN11/00Kan and select plasmids from the BLAST search are presented in Fig. 1. Searches using the BLASTN program (megablast) against plasmid pSN11/00Kan resulted in very few hits of native small kanamycin resistance plasmids in the database. Plasmid pSN11/00Kan carried the identical *aph*(3′)-I gene as that of the pU302S (Accession No. AY333433) and pG7601Kan, and was 92% identical to pU302S at the nucleotide level in the region of RNA I/II (nt 226–946).

Table 1
Open reading frames and their putative protein functions on plasmid pSN11/00Kan.

Gene name	# Amino acids	Position (Start–Stop)[a]	Best BLASTP match		
			Description; (amino acid length); organism/source	Protein identity (identical/total aa)	GenBank accession #[b]
rom	63	c1354–1163	Rom, RNA I modulator protein (63aa); *Salmonella choleraesuis*	98% (61/62)	ref|NP_203136
mobC	115	1393–1740	MbeC mobilization protein (115aa); *E. coli* SMS-3-5	86% (100/115)	ref|YP_001739875
mobA	529	1730–3319	MbeA mobilization protein (524aa); *Salmonella enterica* subsp. *enterica* serovar Heidelberg str. SL476	92% (491/529)	ref|YP_002043968
mobB	186	2418–2978	MbkB (186aa); *E. coli*	75% (140/186)	ref|YP_214177
mobD	77	2985–3218	MobD (77aa); *E. coli*	71% (55/77)	ref|YP_794136
aph(3′)-I	271	4295–5110	Aminoglycoside 3′-phosphortranferase type 1 (271aa); *Salmonella* Typhimurium	100% (271/271)	ref|YP_194807

[a] Number preceded with a "c" indicates that the coding sequence is on the complement strand.
[b] "ref|" preceding the accession number indicate Ref Seq collection of NCBI GenBank.

Table 2
DNA and RNA features on plasmid pSN11/00Kan.

Feature name	Position[a]	Putative function	Note	Reference
RNA I	c329–226	Transcript RNA I, binds to RNA II thus inhibiting primer formation	Based on pU302S	Chen et al. (2007)
RNA II	224–747	Transcript RNA II primer precursor	Based on pU302S	Chen et al. (2007)
oriV	748–750	Origin of replication	Based on pSFD10	Liu et al., 2002
oriT	897–1136	Origin of DNA transfer	Based on pSFD10	Liu et al. (2002)
nic	1016–1017	Relaxation cut site	Based on ColE1	Bastia (1978)
cer (res)	3476–3619	Resolution region; replication termination	Based on pSFD10	Liu et al. (2002)

[a] Number preceded with a "c" indicates that the direction of transcription is on the complement strand.

The *aph*(3′)-I gene was 98% identical at the nucleotide level to that of *E. coli* kanamycin resistance plasmid pUB2380 (Accession No. AJ008006); however, the similarity to pUB2380 is limited to the *rom* gene and part of the *mob* operon (nt 819–2576, 84% identity) and does not extend to the RNA I/II region (Fig. 1). Plasmid pSN11/00Kan showed an extensive area (⩾2 kb) of similarity within the region involved in replication/copy number control (RNA I/II, *ori*V, *ori*T, *rom*) and/or plasmid mobilization (*mobC*, and *mobABD*) to several native *Salmonella* and *E. coli* plasmids. The most extensive region of similarity (spanning essentially the entire plasmid replication and mobilization region, nearly 3 kb in-length) was found with the colicinogenic *E. coli* plasmids pColK-K235 (AY929248; Rijavec et al., 2007; nt 224–3320, 84% identity) and pColD-157 (Y10412; Hofinger et al., 1998; nt 292–694 and 704–3320, 83% identity); and to a lesser extent, with plasmid ColE1 (J01566; Chan et al., 1985; nt 381–2576, 87% identity), plasmid pSMS35_8 from an *E. coli* MR environmental isolate (CP000972; Fricke et al., 2008; nt 383–2576, 87% identity) and two *Salmonella* plasmids *Salmonella* Enteritidis plasmid pC (AY079201; Gregorova et al., 2002; nt 269–2576, 86% identity) and *Salmonella choleraesuis* plasmid pSFD10 (AY048853; Liu et al., 2002; nt 385–2574, 87%

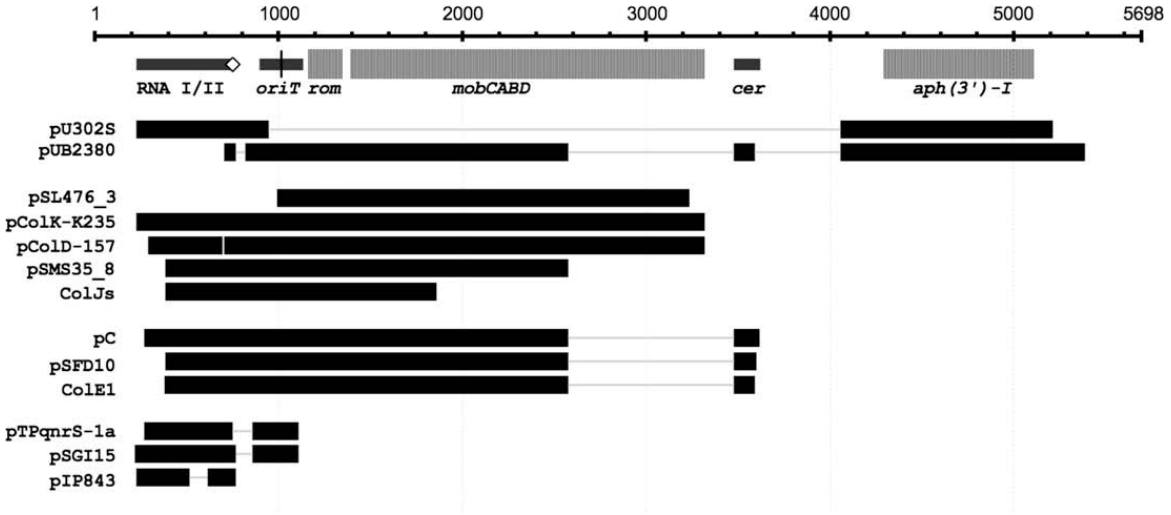

Fig. 1. Regions of similarity of pSN11/00Kan to entries in the GenBank database. Similarity was analyzed using BLASTN program (optimized for megablast). Top: Gene organization of plasmid pSN11/00Kan. Thin gray bars, DNA and RNA features involved in plasmid replication, maintenance and transfer; diamond, *oriV*; striped bars, protein coding regions; black straight line in the *oriT* region indicates the *nic* site; scale in base pair. Bottom: Black bars, regions of similarity between pSN11/00Kan and other plasmids. Details of the plasmids are listed below and were further discussed in the text.

Name	Size (bp)	Accession #	Bacterial strain	Reference
pU302S	3208	AY333433	*Salmonella* Typhimurium strain G8430	Chen et al. (2007)
pUB2380	8561	AJ008006	*Escherichia coli* strain SE53	Direct submission
pSL476_3	3373	CP001119	*Salmonella* Heidelberg strain SL476	Direct submission
pColK-K235	8318	AY929248	*Escherichia coli*	Rijavec et al. (2007)
pColD-157	6675	Y10412	*Escherichia coli* O157:H7 strain CL40	Hofinger et al. (1998)
pSMS35_8	8909	CP000972	*Escherichia coli* SMS-3-5	Fricke et al. (2008)
ColJs	5210	AF282884	*Shigella sonnei*	Smajs and Weinstock (2001)
pC	5269	AY079201	*Salmonella* Enteritidis	Gregorova et al. (2002)
pSFD10	4091	AY048853	*Salmonella choleraesuis* strain 79500	Liu et al. (2002)
ColE1	6646	J01566	*Escherichia coli*	Chan et al. (1985)
pTPqnrS-1a	10066	AM746977	*Salmonella* Typhimurium	Kehrenberg et al. (2007)
pSGI15	2699	FN428572	*Salmonella* Typhimurium	direct submission
pIP843	7086	AY033516	*Klebsiella pneumoniae* strain BM4493	Cao et al. (2002)

identity). Although the identity score to plasmid pSL476_3 from *S. enterica* serovar Heidelberg str. SL476 (CP001119) was the highest (nt 995–3235, 96% identity), the region of similarity was limited to the *rom* gene and the *mobCABD* genes. Plasmid pSN11/00Kan also showed 89% identity to *Shigella sonnei* plasmid ColJs (AF282884; Smajs and Weinstock, 2001) between nucleotide 383–1860 (Fig. 1).

Recent efforts on the characterization of ColE-like plasmids carrying antimicrobial resistance genes have mainly been focused on those carrying quinolone resistance genes or the CMY- or CTX-M-type β-lactamases, but complete plasmid sequences in the database were scarce (Carattoli, 2009). The BLAST search (optimized for megablast) against pSN11/00Kan did not return any plasmids carrying *qnr*, bla_{CMY}, or $bla_{CTX\text{-}M}$ genes. Relevant plasmid sequences were manually retrieved from GenBank and compared to pSN11/00Kan using "Align (bl2seq)" optimized for megablast and showed limited regions of sequence identity (79–90%) restricted to only the RNA I/II and *oriT* region (Fig. 1). The results suggest that these ColE1-like plasmids are distantly related to the plasmids carrying kanamycin resistance genes reported in this study. When a less stringent parameter was applied ("Align" optimized for "somewhat similar sequences (blastn)") the regions of similarity were extended slightly, although with lower degree of identity (69–77%) in the RNA I/II, *oriT* and *cer* regions to several *S.* Typhimurium *qnr* plasmids pSGI15 (FN428572), pTPqnrS-1a (AM746977; Kehrenberg et al., 2007) and pST728/06-2 (EU715253; Wu et al., 2008). The two almost identical plasmids pTPqnrS-1a and pST728/06-2 (isolated from Europe and Taiwan, respectively) also showed limited sequence identity to pSN11/00Kan in the *mob* region (data not shown). Plasmid pTPqnrS-1a carrying *qnrS1* and plasmid pSGI15 carrying *qnrB19*, both isolated in Europe, shared 82% identity in a 900 bp fragment including the RNA I/II and part of the *oriT* (data not shown). Four plasmids carrying bla_{CMY} or $bla_{CTX\text{-}M}$ gene also were found to share limited sequence similarity (~66–73% identity) with pSN11/00Kan: pA172 from *S.* Newport (EU331425; Zioga et al., 2009), pH205 (EU331426; Zioga et al., 2009) and pIP843 (AY033516; Cao et al., 2002) from *Klebsiella pneumoniae*, and pTKH11 from *Klebsiella oxytoca* (Y17716, Wu et al., 1999). Most of these plasmids carried the resistance genes as well as complete insertion sequences (IS*Ecp*1-like or IS903) and carried only a single mobilization gene (*mobB*) or none at all (Cao et al., 2002; Wu et al., 1999; Zioga et al., 2009). All three bla_{CMY}-encoding plasmids (pA172, pH205 and pTKH11) were almost identical with only a few nucleotide differences between each other (Zioga et al., 2009). The CTX-M plasmid pIP843 only showed homology to the CMY plasmids pA172/pH205/pTKH11 in the RNA I/II (75% identity), the *oriT* and *cer* regions (~84–92% identity), other than the nearly identical IS*Ecp*1 IS elements (99% identity).

Our results showed that two 5698 bp Kan^R ColE1-like plasmids from two different *S.* Newport strains (isolated from two different years and different regions in the US) were identical, and the 3208 bp pG7601Kan plasmid from *S.* Typhimurium was also found to be identical to another previously sequenced plasmid pU302S from Typhimurium. This suggests that these ColE1-like plasmids might be prevalent amongst kanamycin resistant strains and that they might be clonal within each *Salmonella* serovar. These Kan^R ColE1-like plasmids are present in high copy number and are stably maintained for ~100 generations without antibiotic selection pressure (data not shown). These plasmids also carry mobilization genes that can be mobilized by other conjugative plasmids of various incompatibility groups including IncFI, IncIa, IncP, and IncW (Finnegan and Sherratt, 1982). Our replicon typing results showed that all three *Salmonella* strains carried additional plasmid replicons: IncFIA, IncFIIA, and IncP in *S.* Typhimurium strain G7601, and IncP and IncA/C in both *S.* Newport isolates SN11/00 and SN02/01, thus the chance of these plasmids being transmitted into other strains is high. With these properties they could pose serious public/animal health concerns should the *Salmonella* strains harboring them become epidemic, thus warranting further large-scale studies to assess the prevalence of ColE1-like plasmids carrying antibiotic resistance determinants. We are currently testing primers for replicon typing to be used singularly, or incorporated into the multiplex primer sets designed by Carattoli and colleagues (Carattoli et al., 2005) to aid in these studies.

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